8 Genomic Adaptation of *Saccharomyces cerevisiae* to Inhibitors for Lignocellulosic Biomass Conversion to Ethanol

ZONGLIN LEWIS LIU[1] AND MINGZHOU SONG[2]

[1]*US Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, Peoria, USA;* [2]*Department of Computer Science, New Mexico State University, Las Cruces, USA*

Introduction

As interest in alternative energy sources rises, the concept of agriculture as an energy producer has become increasingly attractive (Outlaw *et al.*, 2005). Renewable biomass, including lignocellulosic materials and agricultural residues, provide low-cost materials for bioethanol production (Bothast and Saha, 1997; Wheals *et al.*, 1999; Zaldivar *et al.*, 2001).

For economic reasons, dilute acid hydrolysis, which hydrolyses the hemicellulose fraction and increases fibre porosity to allow enzymatic saccharification and fermentation of the cellulose fraction, is commonly used in biomass degradation (Bothast and Saha, 1997; Saha, 2003). However, one major limitation of this method is the generation of numerous by-products and compounds that inhibit microbial growth and subsequent fermentation.

The stress conditions involved in the distinct lignocellulosic biomass conversion process have been a technical barrier in biomass conversion to ethanol. Biomass pre-treatment generates varied harsh conditions, including high temperatures, extreme pH, high substrate concentration, osmotic shifts and toxic compounds that inhibit yeast growth and fermentation. Furfural and 5-hydroxymethylfurfural (HMF) are major inhibitors commonly recognized from biomass pre-treatment. Genetic mechanisms involved in the tolerance of stresses such as those caused by furfural and HMF are unknown, and few yeast strains are available that are tolerant to these inhibitors.

Development of stress-tolerant ethanologenic yeasts is one of the significant challenges for cost-competitive bioethanol production. Recently,

progress has been made in developing more tolerant strains to detoxify furfural and HMF *in situ*. This chapter summarizes current knowledge in this regard and discusses future applications using genomic adaptation for new strain development.

Biomass Conversion Inhibitors

Furfural and HMF derived from biomass pretreatment

More than 100 compounds derived from pre-treatment of biomass substrates have been shown to have potential inhibitory effects on microbial fermentation (Luo *et al.*, 2002). Common inhibitors have been identified and classified into the four groups of aldehydes, ketones, organic acids and phenols (Palmqvist and Hähn-Hägerdal, 2000; Klinke *et al.*, 2004; Liu and Blaschek, 2009). Furfural and 5-hydroxymethylfurfural derived from biomass pre-treatment are among the most significant and potent inhibitors to yeast growth and fermentation (Chung and Lee, 1985; Olsson and Hähn-Hägerdal, 1996; Taherzadeh *et al.*, 2000a).

During biomass degradation by dilute acid treatment, cellulose components are converted to hexoses, and hemicellulose components are converted to pentoses. Furfural and HMF are derived from further dehydration of pentoses and hexoses, respectively (see Fig. 8.1; Dunlop, 1948; Antal *et al.*, 1990, 1991; Larsson *et al.*, 1999; Lewkowski, 2001).

These compounds reduce enzymatic and biological activities, break down DNA, inhibit protein and RNA synthesis and damage yeast cell walls (Sanchez and Bautista, 1988; Khan and Hadi, 1994; Modig *et al.*, 2002; Gorsich and Liu, unpublished data). Most yeasts, including industrial strains, are susceptible to the complexes associated with dilute acid hydrolysis pre-treatment and can be killed by low concentrations of inhibitory complexes (Palmqvist *et al.*, 1999; Taherzadeh *et al.*, 2000a; Martin and Jonsson, 2003; Liu *et al.*, 2004). Additional remediation treatments, including physical, chemical, or biochemical detoxification procedures, are often required to remove these inhibitory compounds and to allow fermentation. However, these additional steps add cost and complexity to the process and generate extra waste products (Martinez *et al.*, 2000; Mussatto and Roberto, 2004).

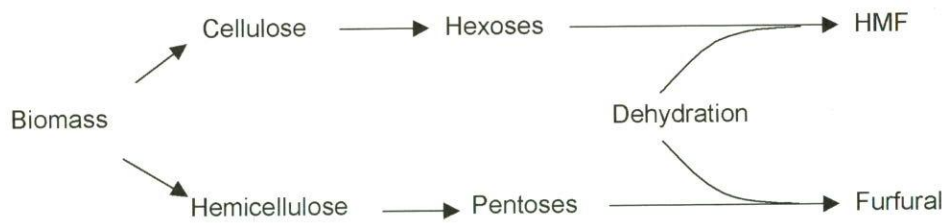

Fig. 8.1. Furfural and HMF derived from biomass pre-treatment. During biomass pre-treatment, cellulose is degraded to hexoses, and hemicellulose to pentoses, from which 5-hydroxymethylfurfural (HMF) and furfural are derived from sugar dehydrations, respectively.

Metabolic conversion pathways of furfural and HMF

Furfural can be converted to furan methanol (FM, furfuryl alcohol) by yeasts (see Fig. 8.2), and it is believed that FM is further reduced to pyromucic acid (Morimota and Murakami, 1967; Nemirovskii and Kostenko, 1991; Villa *et al.*, 1992; Mohsenzadeh *et al.*, 1998; Liu, 2006). Furfural can also be broken down to form formic acid (Palmqvist and Hähn-Hägerdal, 2000). The biotransformation of furfural and HMF by yeasts is due to NADH- and NADPH-coupled enzymes (Palmqvist *et al.*, 1999; Larroy *et al.*, 2002; Liu, 2006; Petersson *et al.*, 2006; Liu *et al.*, 2008b).

In the presence of furfural the intracellular concentration of ATP is low, cell replication is limited and glycerol formation is reduced. Furfural is an electron acceptor and so it can cause a shortage of NADH (Wahlbom and Hähn-Hägerdal, 2002). As furfural reduction competes for NADH, it interferes with cell glycolysis during regeneration of NAD^+. As a result, the presence of furfural can lead to an accumulation of acetaldehyde and a delay in acetate and ethanol production. A reduction in xylitol excretion has also been reported during xylose fermentation when furfural was added to the medium (Wahlbom and Hähn-Hägerdal, 2002). Reduced furfural tolerance was observed with some selective deletion mutants of genes in the pentose phosphate pathway (Gorsich *et al.*, 2005), and these observations can be related to NADPH-dependent reactions involved in pentose phosphate pathways. Enzyme cofactor imbalances also appeared to be affected by furfural.

Unlike furfural, knowledge of HMF conversion has been limited because there has not been a readily available commercial source for an HMF conversion product. Following the furfural conversion route, it has been assumed that HMF is converted to HMF alcohol (Nemirovskii *et al.*, 1989). Recently, an HMF metabolic conversion product was isolated and identified

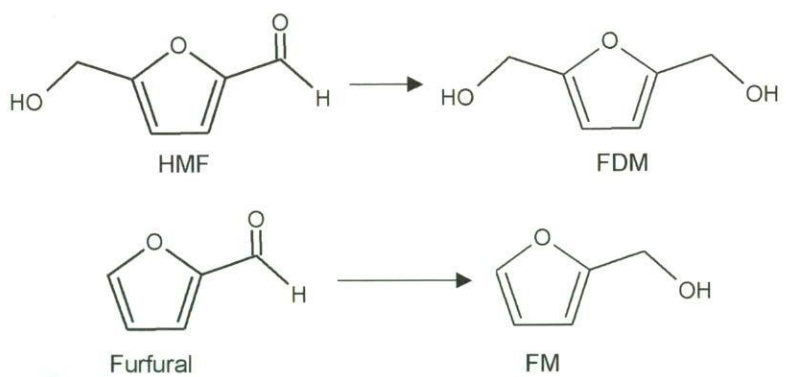

Fig. 8.2. Inhibitor conversion pathways. 5-Hydroxymethylfurfural (HMF) metabolic conversion product is identified as 2,5-bis-hydroxymethylfuran (also called furan-2,5-dimethanol, FDM), with the formula $C_6H_8O_3$ and a molecular mass of 128 by GC-MS and NMR; and furfural is converted to furan methanol (FM) (from Liu *et al.*, 2008a).

as furan-2,5-dimethanol (FDM), also termed 2,5-bis-hydroxymethylfuran (Liu *et al.*, 2004, 2008a; Liu, 2006; Fig. 8.2). FDM has been isolated from cell free culture supernatant, purified and characterized using mass and NMR spectra analysis (Liu *et al.*, 2004). Signals for the aldehyde proton and the asymmetric spectra of HMF were absent when the purified HMF-conversion product was analyzed using NMR, and the NMR spectra were consistent with that of a symmetrical molecule with a furan ring. The metabolite was found to have a composition of $C_6H_8O_3$ and a molecular mass of 128 g/mol (see Fig. 8.2). The characterization of FDM has clarified the existing literature and provides a basis for new studies on mechanisms for the detoxification of the inhibitor.

Yeast Adaptation to Furfural and HMF

Microbial performance is the key for cost-efficient improvement

Fermentation is among the oldest microbial applications in human history. Although a tremendous amount of knowledge has been accumulated through years of experience and development of modern technology, many alternative fermentation processes remain unknown. The economics of fermentation-based bioprocesses rely extensively on the performance of microbial biocatalysts for their industrial application. Development of yeast strains that can efficiently utilize heterogeneous sugars and withstand stress conditions in the bioethanol conversion process is key for sustainable and cost-competitive conversion of lignocellulosic biomass to ethanol. However, many of the industrially interesting microorganisms obtained so far are not sufficiently robust.

Genetically manipulated yeast strains have generally enhanced ethanol fermentation performance, due to improvements in their sugar utilization and enzyme production (Ho *et al.*, 1998; Jeffries and Shi, 1999; Ostergaard *et al.*, 2000; Hähn-Hägerdal *et al.*, 2001). Development of genetically engineered strains with greater tolerance to inhibitors, especially to furfural and HMF, is a promising alternative to the costly traditional inhibitor remediation steps (Liu and Slininger, 2005; Liu *et al.*, 2005). However, development of such strains is hindered due to a lack of understanding of the basic mechanisms underlying stress tolerance in ethanol production by *Saccharomyces cerevisiae*. The fast life cycle and genetic diversity of ethanologenic yeasts are invaluable resources for strain improvement, and an efficient utilization of these characteristics will lead to more cost-effective fermentation and processing in the future.

Dose-dependent response

On a defined medium, yeast strain Y-12632 showed clear dose-dependent cell growth and metabolic conversion activities in response to varied doses of HMF and/or furfural, and cell growth was delayed at concentrations of 10, 30 and 60 mM (Liu *et al.*, 2004). Metabolic transformation of HMF into FDM,

furfural into FM and glucose into ethanol was also delayed with the increase in inhibitor doses (Liu *et al.*, 2004). The lag phase lasted from a few hours to several days, depending upon the concentration of the inhibitors and the strains used. Once cell growth had recovered, cultures inoculated with sublethal doses of the inhibitors were able to utilize glucose and produce ethanol. This demonstrated a clear dose-dependent inhibition of the yeast by furfural and HMF. However, this lag phase was not observed at the higher concentration of 120 mM, where cells were completely repressed and no biological activity or HMF transformation was observed (Liu *et al.*, 2008b).

The duration of the lag phase may be interpreted as a measure of the level of tolerance to furfural and HMF. This suggests that some yeast strains have more effective mechanisms than others to withstand these inhibitors. The prolonged lag phase before the recovery of cell growth could reflect a genetic response, and result in a shift in physiology of the cells adapting to the chemical stress. It has been suggested that certain enzymes may be induced during the lag phase (Kang and Okada, 1973; Liu and Slininger, 2005, 2006), and important metabolic enzymes – including alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase – have been reported to be inhibited by furfural and HMF *in vitro* (Modig *et al.*, 2002). Numerous enzymes have been found to have significantly enhanced expression in the presence of furfural and HMF (Liu *et al.*, 2008a, b), and yeasts are apparently stimulated to undergo an adaptation process in response to HMF during the lag phase (Liu and Slininger, 2006).

Enhanced biotransformation and tolerance

Adaptation of *S. cerevisiae* to furfural and HMF has been observed, and methods used to overcome inhibitory effects have included the use of increased yeast inoculum level, increased biomass or fed-batch mode fermentation (Banerjee *et al.*, 1981; Chung and Lee, 1985; Villa *et al.*, 1992; Taherzadeh *et al.*, 2000b). An adapted *Pichia stipitis* that gave improved ethanol production from hemicellulose hydrolysate has been reported (Nigam, 2001). The dose-dependent yeast response to furfural and HMF has been used to generate strains tolerant to either furfural or HMF by a directed adaptation method (Liu *et al.*, 2005).

Recently, a newly developed strain, Y-50049, has shown tolerance to both furfural and HMF and was able to complete ethanol fermentation in 48 h, while a wild type failed to establish a culture under the same conditions (Liu *et al.*, 2008b). This strain did not require a pre-build biomass but functioned as an initial inoculum to establish a culture. The tolerant strain showed significantly enhanced biotransformation, converting furfural into FM, HMF into FDM and produced a normal yield of ethanol. In contrast, a normal control strain failed to establish a culture in the presence of the inhibitors 48 h after inoculation. This showed that a qualitative change could be derived from evolution by quantitative adaptation, leading to a genetic adaptation in the response to the inhibitors.

Strain improvement by directed evolutionary adaptation

The directed evolution method has been used to obtain numerous strains with improved performance. In addition to the yeast strains tolerant to furfural and HMF (Liu *et al.*, 2004, 2005, 2008b; Liu and Slininger, 2006), a preliminary improvement to inhibitor tolerance was recently obtained for a sugarcane bagasse hydrolysate by adaptation of an engineered xylose utilization strain (Martin *et al.*, 2007). At least two phenotypically different populations have been recovered from a recombinant strain by selection pressures applied during an evolution method (Sonderegger and Sauer, 2003). Under such conditions, multiple mutations appeared necessary to obtain more integrated functional adaptations. Efficient xylose utilization strains of *S. cerevisiae* were obtained through directed evolution with a minimum of recombinant engineering of exogenous xylose isomerase (Kuyper *et al.*, 2005). Strains of *S. cerevisiae* tolerant to HMF and also able to grow on xylose have been obtained in the laboratory through directed evolution methods (Liu *et al.*, unpublished data).

The persistence of specific altered gene expression over time supports the hypothesis that yeasts are stimulated to undergo an adaptation process during the lag phase in response to inhibitors (Liu, 2006; Liu and Slininger, 2006). Directed evolutionary adaptation therefore appears promising for the development of desirable characteristics in ethanologenic yeasts (Kuyper *et al.*, 2005; Liu *et al.*, 2005, 2008a; Liu, 2006). The adaptation approach can be an alternative means of improving microbial strain performance, and such adapted strains may be suitable for further genetic manipulation. Further study in this area can be expected to lead to new strain development.

Adaptation is not a new process in yeast utilization, and success depends upon the genetic potential of the yeast. Earlier studies have shown a link between genetic potential and the ability of a yeast to withstand and transform furfural and HMF. The genetic potential for all of the stress conditions encountered in the bioethanol conversion process has not been experimentally tested. Enrichment of the genetic background of the candidate yeast can be achieved by introducing exogenous gene functions, following proper genetic manipulation and adaptation. Development of tolerant strains with enhanced detoxification using directed enzyme evolution has shown some promising results (Moon and Liu, unpublished data). Enhanced laboratory procedures can significantly speed up evolutionary adaptation to the stress condition and may maintain desirable ethanol production characteristics.

Functional Genomics of Ethanologenic Yeast

Understanding mechanisms of tolerance using functional genomics

Single-gene studies have contributed significantly to our knowledge of gene functions in the past 50 years and will continue to do so in the future. However, the new advances in genomics have revolutionized our understanding and changed our view on yeast-processing events. A biological

process often cannot be explained by a single gene function, and is frequently the result of a complex control system. As thousands of genes in a genome are required to maintain a living yeast system, significant gene alteration can have a significant impact on the responses of other genes in the system. Significant gene interactions and genomic regulatory networks need to be considered for efficient genetic manipulation for strain development (Liu and Slininger, 2005), and a few enhanced functional genes are unlikely to address the challenges encountered in bioethanol conversion.

An understanding of the genomic mechanisms involved in the integration and balance of these functions in individual strains through directed evolutionary adaptation is needed. Such knowledge provides a fundamental insight into the integrated alteration of genome architecture, transcriptional profiling and gene regulatory networks that underline heterogeneous sugar utilization and stress tolerance. This new technology will provide great flexibility and power in the design and development of more desirable and robust biocatalysts for cost-effective and highly productive lignocellulosic conversion to ethanol for the next decade. In order to understand mechanisms of stress tolerance to furfural and HMF, it is necessary to identify key gene functions, gene interaction networks and regulatory elements involved at the genome level.

Yeasts live in ever-changing environments and need constantly to adapt to external stimuli for survival. As documented in numerous reports, yeast adaptation to stress conditions is common and accomplished via a variety of molecular mechanisms (Gasch and Werner-Washburne, 2002; Erasmus *et al.*, 2003). Laboratory strains of yeasts have been used extensively as model organisms in studies of genomic expression profiling with varied environmental stimulants (Gasch *et al.*, 2000; Causton *et al.*, 2001; Brejning *et al.*, 2003; Zhang *et al.*, 2003; Lucau-Danila *et al.*, 2005).

Common stress-tolerant genes have been reported, and the transient expression responses to stimuli appear to be common. Genome expression and transcriptome dynamics to environmental stress and other fermentation stress conditions, including HMF, have also been studied for some industrial yeasts (Chen *et al.*, 2003; Erasmus *et al.*, 2003; James *et al.*, 2003; Devantier *et al.*, 2005; Liu and Slininger, 2006). However, systematic information on the inhibitory stress tolerances involved in the bioethanol conversion process at the genome level is not yet available. Due to the heterogeneity of experimental conditions and a lack of common quality control for multiple microarray experiments, it is impossible to make comparisons between existing studies. Currently, an integrated functional genomic approach has been taken to study furfural and HMF stress tolerance involved in bioethanol conversion and to develop more inhibitor-tolerant strains (Liu and Slininger, 2005).

Quality control issues for gene expression analysis

The significance of the proper application of quality controls cannot be overestimated in ensuring the reliability and reproducibility of microarray

expression data. Quality control has been a very important issue since the emergence of high-throughput gene expression technology (Schena *et al.*, 1995; Brazma *et al.*, 2001; Badiee *et al.*, 2003), and ever-increasing attention has been drawn to concerns over the application of expression data (Baker *et al.*, 2005; Bammler *et al.*, 2005; Larkin *et al.*, 2005). The need for standard controls across different platforms of gene expression analysis has been recognized (Dallas *et al.*, 2005; Etienne *et al.*, 2005; External RNA Control Consortium, 2005; Irizarry *et al.*, 2005).

Six species of exogenous nucleotides have been used as a set of universal external RNA quality controls, which were developed specifically for microbial gene expression analysis across different platforms of microarray and real-time quantitative RT-PCR (Liu and Slininger, 2007). The DNA sequences of these control genes were compared with those in the microbial gene sequence database (Peterson *et al.*, 2001). The selected control genes had no homology or similarity to the yeast genome and bacterial system, and therefore avoided interference with microbial gene expression signals. The linearity of signal intensity of the control genes allowed them to serve as a quantitative calibration and normalization reference for quantitative measurement of gene expression analysis. Using these quality controls, a coefficient of variance can be calculated and analysis of variance can be applied to results (Kerr and Churchill, 2001; Churchill, 2004).

Unlike housekeeping genes usually affected by environmental conditions, these controls demonstrate consistency in mRNA detection independent of environmental factors. Such quality control measurements provide a consistent reference, allow an estimate of variation for microarray experiments, reduce variability and increase the reliability and reproducibility of microarray data. The application of universal quality controls will allow confirmation and comparison of data obtained from different microarray experiments and platforms. In order to generate high-quality data from microarray experiments, it is strongly recommended that the complete length of cDNA populations of labelled probes be evaluated using a microgel or slide gel electrophoresis system (Lage *et al.*, 2002; Liu and Slininger, 2005, 2007). Such a quality control measurement cannot be substituted by quantitative measurements of the probe-using spectrophotometer.

Genomic expression response to furfural and HMF

Yeast genes have been shown to give an immediate response, at least 10 min after exposure to furfural or HMF (Liu and Slininger, 2006). The expression levels of several hundred genes were significantly different for the yeast under HMF stress conditions compared with those of an untreated normal control. These genes demonstrated significant differential expression patterns during a lag phase under HMF stress, and are more likely to be involved in HMF stress tolerance. Thus stress responses are not single gene-controlled events but are an organized global expression response involving multiple genes. Of the genes that were significantly induced, members of the pleiotropic drug

resistance gene family were suggested as playing an important role for cell survival in coping with inhibitor stress (Liu *et al.*, 2006).

Unlike the transient changes reported for laboratory strains, constant functional mRNA expression was observed for ethanologenic yeast in response to the HMF stress during the lag phase. Some genes showed continued enhanced or repressed expression, while others demonstrated significant dynamics of reversed expression (Liu and Slininger, 2006). Genes involved in biological processes, cellular components and molecular function were identified and, of these, some appeared to be HMF- and/or furfural-specific, while others shared functions with those in a core set of common stress genes. Regulatory elements and transcription factors significant for HMF tolerance have been identified (Liu and Sinha, 2006). However, interpretation of some genes was limited by incomplete annotations or lack of known functions. Further studies are needed at the genome level. Many valuable genome resources are available, such as the commonly used *Saccharomyces* Genome Database (Fisk *et al.*, 2006), YEASTRACT (Teixeira *et al.*, 2006), Kyoto Encyclopedia of Genes and Genomics (Kanehisa *et al.*, 2006) and Gene Ontology (The Gene Ontology Consortium, 2000).

Computational modelling of gene regulatory networks for HMF stress

Computational modelling to infer gene regulatory networks (GRN) has recently shown that various potentially interesting gene interactions are involved in inhibitor detoxification (Song and Liu, 2007). Discrete dynamic system models using first-order linear difference equations have been built for the transcriptional interactions among genes in yeast during the earlier exposure to the inhibitor HMF. In a discrete dynamic system model, the expression change rate of a gene is a linear function of the concentrations of potential regulator genes, and one equation is used for each gene. A GRN is derived from a discrete dynamic system model by creating an edge from every potential regulator to each gene it regulates. These models were developed based on mRNA abundance over five time points in the presence or absence of HMF (see Fig. 8.3).

A reconstructed GRN with a subset of 46 gene nodes plus an HMF node showed complex gene interactions under HMF stress (see Fig. 8.4). Forty-six significantly induced expressed genes from the HMF treatment were selected based on ANOVA and cluster analysis, and these were used for the prototype computational model development. This system model captured temporal dependencies among the 46 genes and HMF during the earlier exposure to the inhibitor in the yeast fermentation process.

The system model underlying the GRN is an optimal solution after searching all possible directed graphs with 47 nodes, except that the HMF node is not allowed to have incoming edges and the maximum number of incoming edges for a gene node is, at most, 5. The existence of an edge from *YAP*1 to *DDI1* indicates a temporal dependency of the rate of change in *DDI1* expression on the mRNA level of *YAP1*. The number 1.2e-07, positioned next

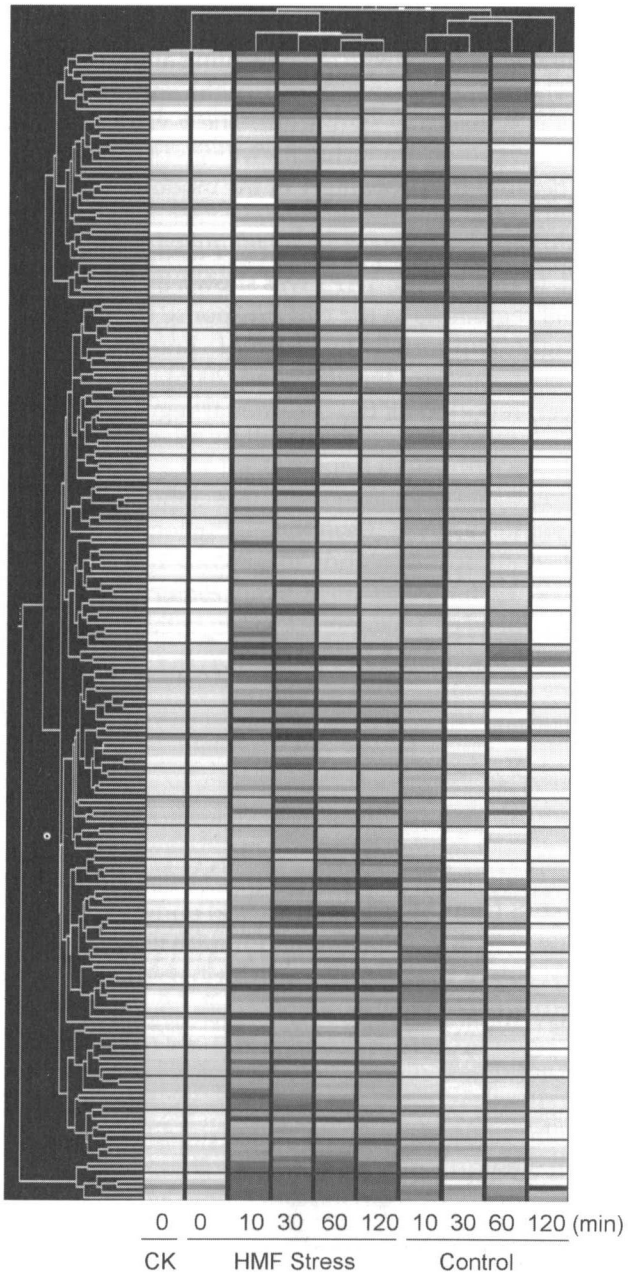

Fig. 8.3. The genomic adaptation to HMF stress. Interactions of significantly expressed genes of ethanologenic yeast *Saccharomyces cerevisiae* under a normal control condition and 5-hydroxymethylfurfural (HMF) stress condition from 0, 10, 30, 60 and 120 min after the treatment, showing significantly induced (blue) and repressed (red) mRNA expression caused by the HMF stress on a defined medium. Yellow colour indicates mRNA equally expressed under different conditions. Varied colours between yellow and red, or yellow and blue, indicate varied relative quantitative measurements of mRNA expression levels in a log scale.

to the edge (see Fig. 8.4), is the *p*-value of this temporal dependency. The original system matrix was stabilized by scaling all eigenvalues by the spectral norm of 3.09. The overall *p*-value, 1.6e-5, of the entire system model indicates that the model is statistically significant. The *p*-value is based on a stringent standard and the resulting model has high levels of consistency with biological observations, because the probability of the model arising by chance is as low as 1.6e-5.

Three known transcription factors, *PDR1*, *PDR3*, and *YAP1*, were examined and, in this subset, *YAP1* was shown by the model to be one of the most influential regulators in the early response to HMF stress (see Fig. 8.4). This is strongly supported by current knowledge and documented experimental observations (Teixeira *et al.*, 2006). For example, the following edges have been reported as transcriptional regulations, including *YAP1* to

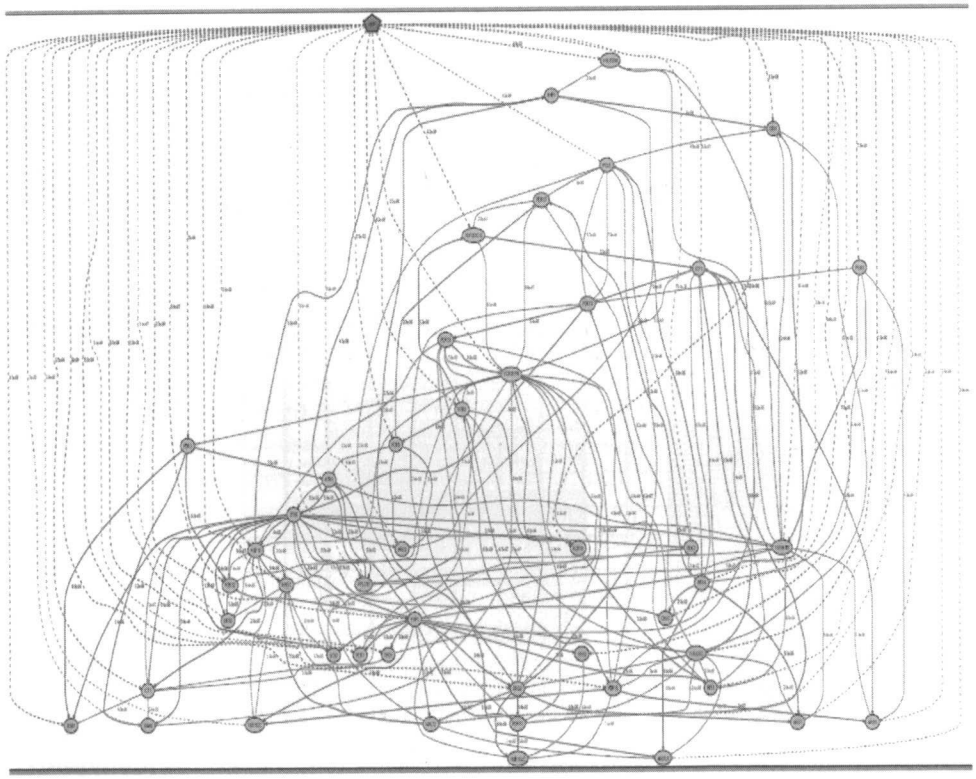

Fig. 8.4. Computation modelling of gene regulatory networks of HMF stress. Temporal interactions for a subset of 46 genes in response to HMF for biomass conversion to ethanol by the ethanologenic yeast. The *p*-values of each edge are displayed. A solid directed edge in green from the first gene node to the second gene node with an arrowhead indicates enhancement of the second gene by the first gene; an edge in red from the first gene node to the second gene node with a solid dot indicates repression of the second gene by the first gene. The dashed edges represent the external influence from HMF to each gene: red for repressing and green for enhancing (from Song and Liu, 2007).

DDI1 (Haugen *et al.*, 2004), *YAP1* to *ATM1* (Haugen *et al.*, 2004), *YAP1* to *GRE2* (Lee *et al.*, 1999), *YAP1* to *SNQ2* (Lee *et al.*, 2002; Lucau-Danila *et al.*, 2005) and *YAP1* to *TPO1* (Lucau-Danila *et al.*, 2005). Four more edges from *YAP1* demonstrated enhancement to *SCS7*, *PDR1*, *PDR11* and *HIS3*, suggesting regulatory roles of *YAP1* to these genes (see Fig. 8.4). The genes *SCS7*, *PDR1*, *PDR11*, and *HIS3* are considered potential transcriptional regulatees of *YAP1* based on sequence motifs (YEASTRACT, 2006). In addition, the transcription factor PDR3 showed a regulatory role to *RSB1* as demonstrated in this model, which is in agreement with and supported by previous documented observations (Devaux *et al.*, 2002). PDR3 also showed enhancement to *SAM3*, *ATM1* and *PDR12*. It is very encouraging that the GRN model developed in this study is highly consistent with the current knowledge, including documented experimental observation and sequence motif-based analysis. More importantly, the model was able to demonstrate statistical significance for the temporal dependencies.

This system model also presented numerous interesting interactions among genes with potential significance. For example, *STE6*, *SNQ2*, *ARG4* and *YOR1* enhanced directly or indirectly for 15, 8, 5 and 4 other genes, respectively. These genes have been observed to be core stress response genes, and many related genes are reported to be involved in survival under HMF stress. Resolution of such interactions could have a significant impact in elucidating the mechanisms of detoxification and stress tolerance caused by HMF. Although they have not been reported, such statistically significant gene interactions presented by this model could be potentially biologically significant in predicting unknown gene interactions. With the high consistency between the system model on *YAP1* presented in this study and current knowledge, it is reasonable to assume potential relationships for associations with significant p-values in this model. Although it is highly homologous with *PDR3*, the common transcription factor *PDR1* did not appear to respond in the same way as *PDR3*, and did not appear to have a significant regulatory role towards the selected subset of genes used in this model.

Another output of the system model is to prescribe the desired system behaviours by applying perturbation to the system. A perturbation can be used to change the concentration level of the inhibitor HMF, silence a subset of genes in the GRN or mutate a subset of genes. In order to increase the tolerance to the inhibitor HMF, one can consider adjusting the influential genes to achieve an effect similar to the transcriptome profile observed in the absence of HMF. This model indicated the following genes to be potentially significant in gene interactions for detoxification and HMF stress tolerance: *STE6* (15/46), *YCR061W* (14/46), *YAP1* (12/46), *YGR035C* (10/46), *SNQ2* (8/46), *HSP10* (7/46) and *YAR066W* (7/46) (see Fig. 8.4). By perturbing these potential regulators, one may exert most control over the expression of other genes, which might be economically desirable. Approaches using linear discrete dynamic system models have shown promising potential to infer complex gene interactions in this computational modelling prototype. More accurate gene regulatory networks will be further defined by continued efforts using additional data and cross-examinations.

Mechanisms of *in situ* detoxification

A recently described furan-2,5-dimethanol preparation procedure can be used as a standard for HPLC metabolic profiling (Liu *et al.*, 2007), and this has in turn allowed studies on mechanisms of *in situ* detoxification of HMF. Studies with the tolerant strain have shown that the furfural and HMF conversion products FM and FDM accumulate in the medium as yeast growth and fermentation is completed. Furfural and HMF are furan derivatives with a furan ring composed of $C_5H_4O_2$ and $C_6H_6O_3$, respectively. Their conversion products FM and FDM contain the furan rings $C_5H_6O_2$ and $C_6H_8O_3$, respectively (see Fig. 8.2).

These furan elements remain intact during the inhibitor conversion process and persist in the medium until the end of the fermentation. The presence of FM and FDM did not affect yeast growth and ethanol yield. Apparently, the aldehyde functional group in furfural and HMF is toxic to yeast but not to the furan ring or the associated alcohol functional groups. Clearly, aldehyde reduction is a mechanism for *in situ* detoxification of furfural and HMF (Liu *et al.*, 2008b).

The toxicity of aldehyde to yeast has been recognized for many years (Leonard and Hajny, 1945). Any potential further reduction or degradation of the furan ring or alcohol groups may not play a significant role for the *in situ* detoxification of furfural and HMF by the yeast. Detoxification of the inhibitors by the yeast did not involve utilization or degradation of the furan compounds, and so terms such as 'furan conversion' or 'furan reduction' that have been used in the literature should not be used in the context of these inhibitor conversions (Liu *et al.*, 2008b). Furthermore, the use of 'furan derivatives' as a general term for 'inhibitors' such as furfural and HMF should be avoided, as FM and FDM are also furan derivatives, and these appear to be either less toxic or non-toxic to yeast.

NAD(P)H-dependent enzymatic activities were observed under furfural and HMF stress conditions (Larroy *et al.*, 2002; Nilsson, *et al.* 2005; Liu, 2006; Petersson *et al.*, 2006; Liu *et al.*, 2008b). Recent studies have shown that aldehyde reduction involves multiple genes with reductase activities and that reduction is not the result of a single gene (Liu *et al.*, 2008b). Numerous genes have been identified that were significantly induced and were responsible for the biotransformation of the inhibitors (Liu, 2006; Liu and Slininger, 2006; Liu *et al.*, 2008b). However, in a yeast culture growing under inhibitor stress, not all of these enzymes are able to function, particularly when the yeast is growing as a batch culture (Liu *et al.*, unpublished data). HMF reduction has been reported to have a cofactor preference for NADPH (Wahlbom and Hähn-Hägerdal, 2002); however, a later study found a different strain of *S. cerevisiae* had a preference for NADH rather than NADPH (Nilsson *et al.*, 2005). The tolerant ethanologenic yeast strain Y-50049, which has an enhanced biotransformation ability, showed enzymatic activities for furfural and HMF reduction with both cofactors (Liu *et al.*, 2008b).

Further studies of selected genes responsible for the de-toxification have

shown that individual enzymes have different cofactor preferences for the reduction of the same inhibitor. For example, in HMF and furfural reduction, *GRE3*- and *ALD4*-encoding enzymes showed strong reductase activity with NADH, while *ADH6* and *ADH7* functioned better with NADPH. In addition, a single functional gene deletion mutant did not significantly change the tolerance of the yeast to the inhibitors. Therefore, the *in situ* detoxification is due to numerous genes rather than to a single one. As for the cofactor preference, certain single genes also have enzyme activities coupled with both cofactors. The whole cell response in detoxification of the inhibitors therefore reflected the collective activities of all functional enzymes (Liu *et al.*, 2008b), and the reduction of furfural and HMF by multiple enzymes with reductase activities is coupled by both NADH and NADPH. The reduction of furfural and HMF therefore compete for NADH and can inhibit glycolysis.

It was observed that in the presence of these inhibitors, glucose was not utilized until appropriate furfural and/or HMF reduction levels had been reached (Liu *et al.*, 2004), and synergistic inhibition by furfural and HMF has been recognized during the extended lag phase of cell growth (Larsson *et al.*, 1999; Taherzadeh *et al.*, 2000a; Wahlbom and Hähn-Hägerdal, 2002; Liu *et al.*, 2004). For normal cell growth, NAD^+ needs to be regenerated from NADH to enable continued glycolysis. In the presence of the inhibitors, furfural and/or HMF can dominate the competition for NADH when they are at higher concentrations and so delay glycolysis. Once the inhibitors have been converted, glucose utilization can occur (Liu *et al.*, 2004).

It is likely that, with the conversion of furfural into FM and HMF into FDM, NAD^+ regeneration becomes freely available and so allows glucose oxidation in glycolysis. Synergistic competition of NADPH also affects biosynthesis pathways and, as a result, the metabolic process can also be significantly altered and delayed in the presence of the inhibitors. In addition to the toxicity of the inhibitors causing cell damage, furfural and HMF will also affect the cellular redox balance.

Conclusion

Dose-dependent inhibition allows for the potential adaptation of ethanologenic yeast so that it is able to transform the inhibitors furfural and HMF into the less toxic compounds of FM and FDM, respectively. The isolation and identification of the HMF metabolic conversion end product as FDM has clarified existing knowledge and provides a basis for metabolic profiling studies of yeast during inhibitor stress tolerance. A genomic approach is needed for efficient improvement of ethanologenic yeast performance. For high-throughput genomic expression studies, the proper application of quality control measurement is critical in ensuring the reliability and reproducibility of expression data, and to confirm and compare data. Gene expression responses of the ethanologenic yeast to furfural and HMF stress during the fermentation were not transient, and the adaptation to furfural

and HMF was a continued dynamic process involving multiple genes. A comprehensive and updated yeast database will allow better global transcriptome profiling of the ethanologenic yeast and provide additional insights into the complexity of adaptation to inhibitor stress.

However, a great deal of information remains unknown and there is only limited functional annotation for some of the significant genes involved in the adaptation. Challenges remain to assign complete functions, draw meaningful conclusions from the complex relationships and assess biological confirmations of gene regulatory networks. Global transcriptome profiling of the tolerant strains is under investigation, and key functional genes and the relevant regulatory components responsible for the biotransformation and de-toxification of the inhibitor will be characterized. A more accurate global account of the genomic mechanism on inhibitor detoxification and tolerance of ethanologenic yeast can be expected from computational modelling of gene regulatory networks. Multiple gene-mediated aldehyde reduction has been demonstrated as a mechanism for the *in situ* detoxification of furfural and HMF. Studies on genomic mechanism of stress tolerance to furfural, HMF and the inhibitory complex involved in bioethanol conversion will be further elucidated to aid more robust strain design and development in the future.

Directed evolutionary genomic adaptation focused on the improvement of specific molecular functions and metabolic dynamics is a powerful means for the improvement and development of desirable strains. Such technology, combined with traditional genetic studies, will bring us to a new horizon in the understanding of ethanologenic yeast. A comprehensive genomic engineering approach will allow us to meet the challenges for efficient lignocellulosic biomass conversion to ethanol into the next decade and beyond.

Acknowledgement

This study was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grant number 2006-35504-17359.

References

Antal, M.J., Mok, W.S.L. and Richards, G.N. (1990) Mechanism of formation of 5-(hydroxymethyl)-2-furaldehyde from D-fructose and sucrose. *Carbohydrate Research* 199, 91–109.

Antal, M.J., Leesomboon, T., Mok, W.S. and Richards, G.N. (1991) Mechanism of formation of 2-furaldehyde from D-xylose. *Carbohydrate Research* 217, 71–85.

Badiee, A., Eiken, H.G., Steen, V.M. and Lovlie, R. (2003) Evaluation of five different cDNA labeling methods for microarrays using spike controls. *BMC Biotechnology* 3, 23.

Baker, S.C., Bauer, S.R., Beyer, R.P., Brenton, J.D., Bromley, B., Burrill, J. *et al.* (2005) The external RNA controls consortium: a progress report. *Nature Methods* 2, 731–734.

Bammler, T., Beyer, R.P., Bhattacharya, S., Boorman, G.A., Boyles, A., Bradford, B.U. *et al.* (2005) Standardizing global gene expression analysis between laboratories and across platforms. *Nature Methods* 2, 351–356.

Banerjee, N., Bhatnagar, R. and Viswanathan, L. (1981) Development of resistance in *Saccharomyces cerevisiae* against inhibitory effects of Browning reaction products. *Enzyme and Microbial Technology* 3, 24–28.

Bothast, R. and Saha, B. (1997) Ethanol production from agricultural biomass substrate. *Advances in Applied Microbiology* 44, 261–286.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoeckert, C. *et al.* (2001) Minimum information about a microarray experiment (MIAME) – toward standards for microarray data. *Nature Genetics* 29, 365–371.

Brejning, J., Jespersen, L, and Arneborg, N. (2003) Genome-wide transcriptional changes during the lag phase of *Saccharomyces cerevisiae*. *Archives of Microbiology* 179, 278–294.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I., True, H.L., Lander, E.S. and Young, R.A. (2001) Remodeling of yeast genome expression in response to environmental changes. *Molecular Biology of the Cell* 12, 323–337.

Chen, D., Toone, M.W., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N. and Bahler, J. (2003) Global transcriptional responses of fission yeast to environmental stress. *Molecular Biology of the Cell* 14, 214–229.

Chung, I.S. and Lee, Y.Y. (1985) Ethanol fermentation of crude acid hydrolyzate of cellulose using high-level yeast inocula. *Biotechnology and Bioengineering* 27, 308–315.

Churchill, G.A. (2004) Using ANOVA to analyze microarray data. *BioTechniques* 37, 173–177.

Dallas, P.B., Gottardo, N.G., Firth, M.J., Beesley, A.H., Hoffmann, K., Terry, P.A., Freitas, J.R., Boag, J.M., Cummings, A.J. and Kees, U.R. (2005) Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR – how well do they correlate? *BMC Genomics* 6, 59.

Devantier, R., Pedersen, S. and Olsson, L. (2005) Transcription analysis of *S. cerevisiae* in VHG fermentation. *Industrial Biotechnology* 1, 51–63.

Devaux, F., Carvajal, E., Moye-Rowley, S. and Jacq, C. (2002) Genome-wide studies on the nuclear PDR3-controlled response to mitochondrial dysfunction in yeast. *FEBS Letters* 515(1–3), 25–28.

Dunlop, A.P. (1948) Furfural formation and behavior. *Industrial Engineering and Chemistry* 40, 204–209.

Erasmus, D., van der Merwe, G. and van Vuure, H (2003) Genome-wide expression analysis: metabolic adaptation of *Saccharomyces cerevisiae* to high sugar stress. *Yeast Research* 3, 375–399.

Etienne, W., Meyer, M.H., Peppers, J. and Meyer Jr, R.A. (2005) Comparison of mRNA gene expression by RT-PCR and DNA microarray. *BioTechniques* 36, 618–626.

External RNA Controls Consortium (2005). Proposed methods for testing and selecting the ERCC external RNA controls. *BMC Genomics* 6, 150.

Fisk, D.G., Ball, C.A., Dolinski, K., Engel, S.R., Hong, E.L., Issel-Tarver, L., Schwartz, K., Sethuraman, A., Botstein, D. and Cherry, J.M. (2006) *Saccharomyces cerevisiae* S288C genome annotation: a working hypothesis. *Yeast 23*, 857–865.

Gasch, A. and Werner-Washburne, M, (2002) The genomics of yeast response to environmental stress and starvation. *Functional and Integrated Genomics* 2, 181–192.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D. and Brown, P.O. (2000) Genomic expression program in the response of yeast cells to environmental changes. *Molecular Biology of the Cell* 11, 4241–4257.

Gorsich, S.W., Dien, B.S., Nichols, N.N., Slininger, P.J., Liu, Z.L. and Skory, C.D. (2005) Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes

ZWF1, *GND1*, *RPE1*, and *TKL1* in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* 71, 339–349.

Hähn-Hägerdal, B., Wahlbom, F., Gárdony, M., Van Zyl, W., Otero, R. and Jönsson, L. (2001) Metabolic engineering of *Saccharomyces cerevisiae* for xylose utilization. *Advances in Biochemical Engineering and Biotechnology* 73, 53–84.

Haugen, A.C., Kelley, R., Collins, J.B., Tucker, C.J., Deng, C., Afshari, C.A., Brown, J.M., Ideker, T. and van Houten, B. (2004) Integrating phenotypic and expression profiles to map arsenic-response networks. *Genome Biology* 5(12), R95.

Ho, N., Chen, Z. and Brainard, A. (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. *Applied and Environmental Microbiology* 64, 1852–1859.

Irizarry, R.A., Warren, D., Spencer, F., Kim, I.F., Biswal, S., Frank, B.C. *et al.* (2005) Multiple-laboratory comparison of microarray platforms. *Nature Methods* 2, 345–350.

James, T.C., Campbell, S., Donnelly, D. and Bond, U. (2003) Transcription profile of brewery yeast under fermentation conditions. *Journal of Applied Microbiology* 94, 432–448.

Jeffries, T. and Shi, Q. (1999) Genetic engineering for improved fermentation by yeasts. *Advances in Biochemical Engineering and Biotechnology* 65, 117–161.

Kanehisa, M., Goto, S., Hattori, M., Aoki-Kinoshita, K.F., Itoh, M., Kawashima, S., Katayama, T., Araki, M. and Hirakawa, M. (2006) From genomics to chemical genomics: new developments in KEGG. *Nucleic Acids Research* 34, D354–D357.

Kang, S. and Okada, H. (1973) Alcohol dehydrogenase of *Cephalosporium* sp. induced by furfural alcohol. *Journal of Fermentation Technology* 51, 118–124.

Kerr, M.K. and Churchill, G.A. (2001) Statistical design and the analysis of gene expression microarray data. *Genetic Research* 77, 123–128.

Khan, Q. and Hadi, S. (1994) Inactivation and repair of bacteriophage lambda by furfural. *Biochemistry and Molecular Biology International* 32, 379–385.

Klinke, H.B., Thomsen, A.B. and Ahring, B.K. (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Applied Microbiology and Biotechnology* 66, 10–26.

Kuyper, M., Toirkens, M.J., Diderich, J.A., Winkler, A.A., van Dijken, J.P. and Pronk, J.T. (2005) Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *FEMS Yeast Research* 5, 925–934.

Lage, J.M., Hamann, S., Gribanov, O., Leamon, J.H., Pejovic, T. and Lizardi, P.M. (2002) Microgel assessment of nucleic acid integrity and labeling quality in microarray experiments. *BioTechniques* 32, 312–314.

Larkin, J.E., Frank, B.C., Gavras, H., Sultana, R. and Quackenbush, J. (2005) Independence and reproducibility across microarray platforms. *Nature Methods* 2, 337–344.

Larroy, C., Fernadez, M.R., Gonzalez, E., Pares, X. and Biosca, J.A. (2002) Characterization of the *Saccharomyces cerevisiae* YMR318C (ADH6) gene product as a broad specificity NADPH-dependent alcohol dehydrogenase: relevance in aldehyde reduction. *Biochemical Journal* 361, 163–172.

Larsson, S., Palmqvist, E., Hähn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G. and Nilvebrant, N. (1999) The generation of inhibitors during dilute acid hydrolysis of softwood. *Enzyme and Microbial Technology* 24, 151–159.

Lee, J., Godon, C., Lagniel, G., Spector, D., Garin, J., Labarre, J. and Toledano, M.B. (1999) *Yap1* and *Skn7* control two specialized oxidative stress response regulons in yeast. *Journal of Biological Chemistry* 274(23), 16040–16046.

Lee, T., Rinaldi, N., Robert, F., Odom, D., Bar-Joseph, Z., Gerber, G. *et al.* (2002) Transcriptional regulatory networks in *Saccharomyces cerevisiae*. *Science*. 298(5594), 763–764.

Leonard, R.H. and Hajny, G.J. (1945). Fermentation of wood sugars to ethyl alcohol. *Industrial Engineering and Chemistry* 37, 390–395.

Lewkowski, J. (2001) Synthesis, chemistry and applications of 5-hydroxymethylfurfural and its derivatives. *Arkivoc* 1, 17–54.

Liu, Z.L. (2006) Genomic adaptation of ethanologenic yeast to biomass conversion inhibitors. *Applied Microbiology and Biotechnology* 73, 27–36.

Liu, Z.L. and Blaschek, H.P. (2009) Biomass conversion inhibitors and *in situ* detoxification. In: Vertès, A.A., Blaschek, H.P., Yukawa, H. and Qureshi, N. (eds) *Biomass to Biofuels*. John Wiley & Sons, Chichester, UK <In press>.

Liu, Z.L. and Sinha, S. (2006) Transcriptional regulatory analysis reveals PDR3 and GCR1 as regulators of significantly induced genes by 5-hydroxymethylfurfrual stress involved in bioethanol conversion for ethanologenic yeast *Saccharomyces cerevisiae*. *Microarray Gene Expression Data Society Meeting* 9, 119.

Liu, Z.L. and Slininger, P.J. (2005) Development of genetically engineered stress-tolerant ethanologenic yeasts using integrated functional genomics for effective biomass conversion to ethanol. In: Outlaw, J., Collins, K. and Duffield, J. (eds) *Agriculture as a Producer and Consumer of Energy*. CAB International, Wallingford, UK, pp. 283–294.

Liu, Z.L. and Slininger, P.J. (2006) Transcriptome dynamics of ethanologenic yeast in response to 5-hydroxymethylfurfural stress related to biomass conversion to ethanol. In: Mendez-Vilas, A. (ed.) *Modern Multidisciplinary Applied Microbiology: Exploiting Microbes and Their Interactions*. Wiley-VCH, Weinheim, Germany, pp. 679–684.

Liu, Z.L. and Slininger, P.J. (2007) Universal external RNA controls for microbial gene expression analysis using microarray and qRT-PCR. *Journal of Microbiological Methods* 68, 486–496.

Liu, Z.L, Slininger, P.J., Dien, B.S,, Berhow, M.A., Kurtzman, C.P. and Gorsich, S.W. (2004) Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. *Journal of Industrial and Microbiological Biotechnology* 31, 345–352.

Liu, Z.L., Slininger, P.J. and Gorsich, S.W. (2005) Enhanced biotransformation of furfural and 5-hydroxy methylfurfural by newly developed ethanologenic yeast strains. *Applied Biochemistry and Biotechnology* 121–124, 451–460.

Liu, Z.L., Slininger, P.J. and Andersh, B.J. (2006) Induction of pleiotropic drug resistance gene expression indicates important roles of PDR to cope with furfural and 5-hydroxymethylfurufral stress in ethanologenic yeast. In: *27th Symposium on Biotechnology and. Fuels Chemistry*, Denver, Colorado, 1–4 May 2005, p. 169.

Liu, Z.L., Saha, B.C. and Slininger, P.J. (2008a) Lignocellulosic biomass conversion to ethanol by *Saccharomyces*. In: Wall, J., Harwood, C. and Demain, A. (eds) *Bioenergy*. ASM Press, Washington, DC.

Liu, Z.L., Moon, J., Andersh, B.J., Slininger, P.J. and Weber, S. (2008b) Multiple gene-mediated aldehyde reduction is a mechanism of *in situ* detoxification of furfural and HMF by ethanologenic yeast *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* 81(4), 743–753.

Lucau-Danila, A., Lelandais, G., Kozovska, Z., Tanty, V., Delaveau, T., Devaux, F. and Jacq, C. (2005) Early expression of yeast genes affected by chemical stress. *Molecular Cell Biology* 25, 1860–1868.

Luo, C., Brink, D. and Blanch, H. (2002) Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol. *Biomass and Bioenergy* 22, 125–138.

Martin, C. and Jonsson, L. (2003) Comparison of the resistance of industrial and laboratory strains of *Saccharomyces* and *Zygosaccharomyces* to lignocellulose-derived fermentation inhibitors. *Enzyme and Microbial Technology* 32, 386–395.

Martin, C., Marcelo, M., Almazan, O. and Jonsson, L.J. (2007) Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. *Bioresource Technology* 98, 1767–1773.

Martinez, A., Rodriguez, M.E., York, S.W., Preston, J.F. and Ingram, L.O. (2000) Effect of $Ca(OH)_2$ treatments ('Overliming') on the composition and toxicity of bagasse hemicellulose hydrolyzates. *Biotechnology and Bioengineering* 69, 526–536.

Modig, T., Liden, G. and Taherzadeh, M. (2002) Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. *Biochemical Journal* 363, 769–776.

Mohsenzadeh, M., Saupe-Thies, W., Sterier, G., Schroeder, T., Francella, F., Ruoff, P. and Rensing, L. (1998) Temperature adaptation of house keeping and heat shock gene expression in *Neurospora crassa*. *Fungal Genetics and Biology* 25, 31–43.

Morimoto, S. and Murakami, M. (1967) Studies on fermentation products from aldehyde by microorganisms: the fermentative production of furfural alcohol from furfural by yeasts (part I). *Journal of Fermentation Technology* 45, 442–446.

Mussatto, S.I. and Roberto, I.C. (2004) Alternatives for detoxification of dilute-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. *Bioresource Technology* 93, 1–10.

Nemirovskii, V. and Kostenko, V. (1991) Transformation of yeast growth inhibitors which occurs during biochemical processing of wood hydrolysates. *Gidroliz Lesokhimm Prom-st* 1, 16–17.

Nemirovskii, V., Gusarova, L., Rakhmilevich, Y., Sizov, A. and Kostenko, V. (1989) Pathways of furfurol and oxymethyl furfurol conversion in the process of fodder yeast cultivation. *Biotekhnologiya* 5, 285–289.

Nigam, J. (2001) Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*. *Journal of Biotechnology* 87, 17–27.

Nilsson, A., Gorwa-Grauslund, M.F., Hähn-Hägerdal, B. and Liden, G. (2005) Cofactor dependence in furan reduction by *Saccharomyces cerevisiae* in fermentation of acid-hydrolyzed lignocellulose. *Applied and Environmental Microbiology* 71, 7866-7871.

Olsson, L. and Hähn-Hägerdal, B. (1996) Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme and Microbial Technology* 18, 312–331.

Ostergaard, S., Olsson, L. and Nielsen, J. (2000) Metabolic engineering of *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews* 64, 34–50.

Outlaw, J., Collins, K.J. and Duffield, J.A. (2005) *Agriculture as a Producer and Consumer of Energy*. CABI Publishing, Wallingford, UK, 345 pp.

Palmqvist, E. and Hähn-Hägerdal, B. (2000) Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. *Bioresource Technology* 74, 25–33.

Palmqvist, E., Almeida, J. and Hähn-Hägerdal, B. (1999) Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. *Biotechnology and Bioengineering* 62, 447–454.

Peterson, J., Umayam, L.A., Dickinson, T., Hickey, E.K. and White, O. (2001) The comprehensive microbial resource. *Nucleic Acids Research* 29, 123–125.

Petersson, A., Almeida, J.R.M., Modig, T., Karhumaa, K., Hähn-Hägerdal, B., Gorwa-Grauslund, M.F. and Liden, G. (2006) A 5-hyfroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae ADH6* gene conveys HMF tolerance. *Yeast* 23, 455–464.

Saha, B. (2003) Hemicellulose bioconversion. *Journal of Industrial Microbiology and Biotechnology* 30, 279–291.

Sanchez, B. and Bautista, J. (1988) Effects of furfural and 5-hydroxymethylfurfrual on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guilliermondii*. *Enzyme and Microbial Technology* 10, 315–318.

Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467–470.

Sonderegger, M. and Sauer, U. (2003) Evolutionary engineering *of Saccharomyces cerevisiae* for anaerobic growth on xylose. *Applied and Environmental Microbiology* 69, 1990–1998.

Song, M. and Liu, Z.L. (2007) A linear discrete dynamic system model for temporal gene interaction and regulatory network influence in response to bioethanol conversion inhibitor HMF for ethanologenic yeast. *Lecture Notes in Bioinformatics* 4532, 77–95.

Taherzadeh, M., Gustafsson, L. and Niklasson, C. (2000a) Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* 53, 701–708.

Taherzadeh, M., Niklasson, C. and Liden, G. (2000b) On-line control of fed-batch fermentation of dilute-acid hydrolysates. *Biotechnology and Bioengineering* 69, 330–338.

Teixeira, M., Monteirom C.P., Jain, P., Tenreiro, S., Fernandes, A.R., Mira, N.P., Alenquer, M., Freitas, A.T., Oliveira, A.L. and Sá-Correia, I. (2006) The YEASTRACT database: a tool for the analysis of transcription regulatory associations in *Saccharomyces cerevisiae*. *Nucleic Acids Research* 34, D446–D451.

The Gene Ontology Consortium (2000) *Nature Genetics* 25, 25–29.

Villa, G.P., Bartroli, R., Lopez, R., Guerra, M., Enrique, M., Penas, M., Rodriquez, E., Redondo, D., Iglesias, I. and Diaz, M. (1992) Microbial transformation of furfural to furfuryl alcohol by *Saccharomyces cerevisiae*. *Acta Biotechnologia* 12, 509–512.

Wahlbom, C.F. and Hähn-Hägerdal, B. (2002) Furfural, 5-hydroxymethylfurfrual, and acetone act as external electron acceptors during anaerobic fermentation of xylose in recombinant *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* 78, 172–178.

Wheals, A.E., Basso, L.C., Alves, D.M. and Amorim, H.V. (1999) Fuel ethanol after 25 years. *Trends in Biotechnology* 17, 482–487.

YEAst Search for Transcriptional Regulators and Consensus Tracking (YEASTRACT) (2006) http://www.yeastract.com (accessed 12 September 2006).

Zaldivar, J., Nielsen, J. and Olsson, L. (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Applied Microbiology and Biotechnology* 56, 17–34.

Zhang, W., Needham, D.L., Coffin, M., Rooker, A., Hurban, P., Tanzer, M.M. and Shuster, J.R. (2003) Microarray analysis of the metabolic response of *Saccharomyces cerevisiae* to organic solvent dimethyl sulfoxide. *Journal of Industrial Microbiology and Biotechnology* 30, 57–69.